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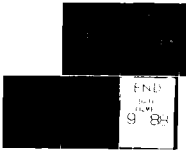

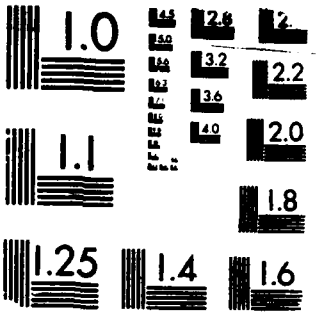

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CLONING AND PRODUCTION OF HUMAN ACETYLCHOLINESTERASE

Final [redacted] Report
May 1982 - April 1984

H. VASKEN APOSHIAN

January 15, 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

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The goal of this work was to clone by DNA recombinant technology the gene for acetylcholinesterase (EC 3.1.1.7) from a human source in order to be able to produce large amounts of pure enzyme under the direction of a gene from a single source. Unfortunately, our attempts were unsuccessful. Lack of success was probably due to the acetylcholinesterase mRNA being present in such small amounts and available techniques not being good enough to detect such minute amounts of messenger. Human neuroblastoma SK-N-SH cells were grown in culture and used in these studies. In retrospect, it appears that seeking cells that produce more than normal amounts of human acetylcholinesterase (HACE) and using modern protein purification procedures might be a better approach to obtain HACE.

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SUMMARY

The goal of this work was to clone by DNA recombinant technology the gene for acetylcholinesterase (EC 3.1.1.7) from a human source in order to be able to produce large amounts of pure enzyme under the direction of a gene from a single source. Unfortunately, our attempts were unsuccessful. Lack of success was probably due to the acetylcholinesterase mRNA being present in such small amounts and available techniques not being good enough to detect such minute amounts of messenger. Human neuroblastoma SK-N-SH cells were grown in culture and used in these studies. In retrospect, it appears that seeking cells that produce more than normal amounts of human acetylcholinesterase (HACE) and using modern protein purification procedures might be a better approach to obtain HACE.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Labortory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

The investigator(s) have abided by the National Institutes of Health, Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

TABLE OF CONTENTS

Summary	2
Foreword	3
Introduction	5
Acetylcholinesterase Activity in Five Cell Lines	5
mRNA Isolation	5
Translation Attempts	6
Polysome Preparation	6
Monoclonal Antibody	7
Preparation of Specific mRNA	7
Purification and Characterization of Monoclonal Antibody from HB-72 Hybridoma	8
Double Immunodiffusion	8
Immunoprecipitation	8
Specificity	8
Comments	9
References	11
Distribution List	12

List of Tables

Table I	Comparison of AChE in Neuroblastoma Cell Lines	10
Table II	Effect of Inhibitors on AChE Activity	10

Introduction

The availability of large amounts of highly purified human acetylcholinesterase (HACE) would be of great aid to research designed to seek new antidotes and new treatments for the so-called nerve gases. At the time these studies were commenced, the logical approach to obtain the large amounts appeared to be by genetic engineering technology. It was our goal to clone the genes for HACE. We were unsuccessful. In retrospect it appears that seeking cells that produce more than normal amounts of HACE and using modern protein purification procedures might be a better approach to obtaining HACE.

Five established cell lines of neuroblastoma, designated LAN-1, LAN-2, SK-N-SH, SK-N-MC, and IMR-32, were first grown and assayed for acetylcholinesterase (AChE).

In order to assay, medium was removed, and cells were processed according to Massoulie and Bon, 1982 (1). Activity was measured using a modification of the method of Rotondo and Fambrough (2) with [^{14}C]acetylcholine as a substrate. Aliquots of cell extract were incubated with 1.2 mM acetylcholine in 100 mM sodium phosphate buffer, pH 7. The reaction was terminated with 50 mM glycine-HCl buffer, pH 2.5. The radioactive product of the reaction, [^{14}C]acetate, was extracted into the scintillation mixture and counted directly. Under the conditions of the reaction, the assay was linear with time and enzyme concentration.

Acetylcholinesterase Activity in Five Cell Lines

A comparison of AChE activity in the five different neuroblastoma cell lines showed that SK-N-SH cells consistently exhibited the highest AChE activity (Table I). The specificity of this reaction was tested with two esterase inhibitors. 1,5-bis-(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284c51) inhibits true AChE selectivity, whereas tetraisopropyl-pyrophosphoramide (IsoOMPA) is an inhibitor of butyryl- or pseudocholinesterase. Ninety-five percent of the activity of SK-N-SH neuroblastoma is true AChE, with no detectable pseudocholinesterase activity (Table II). Therefore, SK-N-SH neuroblastoma was chosen as the best candidate for the isolation of AChE mRNA.

mRNA Isolation

Procedure No. 1. The medium of neuroblastoma cells growing in log phase was supplemented with 50 µCi of [5,6-^{3}H]uridine for 1-2 days. Cells were harvested and the cytoplasmic membranes disrupted in 10 mM Tris/10 mM NaCl/1% Nonidet P-40/10 mM vanadylribonucleoside complexes buffer, pH 7.5. Nuclei were pelleted, the supernatant collected, and buffer added to a final concentration of 0.4 M Na acetate/1% sodium dodecyl sulfate/10 mM EDTA at 4°C. This homogentate was extracted with phenol/chloroform/isoamyl alcohol (24:1:1), and RNA was precipitated from the aqueous phase at -20°C by the addition of 2.5 volumes of ethanol. mRNA was isolated by oligo(dT)-cellulose chromatography as described below. Linear 15-30% sucrose gradients were prepared in 0.1 M NaCl/10 mM Tris-HCl/1 mM EDTA buffer, pH 7.5. Gradients were 10 ml over a 0.3-ml, 50% sucrose pad. A 0.3-ml RNA sample in the same buffer was layered on each gradient. Gradients were centrifuged in a Beckman SW-41 rotor (Beckman Instruments, Inc., Palo Alto, California 94304) at 100,000 xg at 21-23°C for 16 hr. The bulk of mRNA isolated in this manner from neuroblastoma forms a broad

band which peaks at 18 S. Two prominent minor peaks, one at 28 S and a heavier species, were also present. Although this procedure of isolation proved adequate for isolation of mRNA, it did not yield a preparation which translated in an in vitro system. Therefore, another isolation scheme was used.

Procedure No. 2. Plates (approximately 10 x 150 mm), containing subconfluent SK-N-SH neuroblastoma were harvested by washing with Dulbecco's buffered saline solution lacking Mg^{2+} and Ca^{2+}. Cells were washed twice by centrifugation for 1 min at 150 x g at 4°C. Ten volumes of 6 M guanidium thiocyanate/5 mM sodium citrate/0.1 M β-mercaptoethanol and 0.5% N-lauroylsarcosine buffer, pH 7.0, were added to the cell pellet, and the DNA was sheared by forcing the viscous mass through a hypodermic fitted with an 18-gauge needle. One gram of CsCl was added to each 2.5 ml of homogenate. This preparation was then layered on 5.7 M CsCl cushions and centrifuged in a Beckman centrifuge at 210,000 xg for 17 hr at 20°C in an SW-41 swinging bucket rotor in polyallomer tubes. The RNA-containing pellet was dissolved in 10 mM Tris/4 mM EDTA/1% sodium dodecyl sulfate , pH 7.5, and then extracted with an equal volume of chloroform/butanol (4:1). The organic phase was reextracted with buffer and aqueous phases were combined. Then 0.1 volume of 3 M sodium acetate and 2.2 volumes of absolute ethanol were added, and RNA was precipitated at -20°C overnight. RNA was collected by centrifugation. The RNA pellet was suspended in sterile 20 mM Tris/0.5 M NaCl/1 mM EDTA, pH 7.6, and applied to an oligo (dT)-cellulose (type III, Collaborative Research, Inc., Bedford, MA 01730) chromatography column equilibrated with the same buffer. After extensive washing, the poly(A)$^+$-containing mRNA was eluted by washing the column with buffer lacking salt. The mRNA fraction was then brought to 0.5 M with NaCl and passed through an oligo-(dT)-cellulose column a second time under identical conditions. The yield of poly(A)$^+$ mRNA was 1-3% of total RNA.

Translation Attempts

A total poly(A)$^+$ mRNA from SK-N-SH neuroblastoma was translated using a commercial rabbit reticulocyte lysate system (Bethesda Research Laboratories, Bethesda, MD). mRNA was brought up in sterile distilled water. The optimum magnesium concentration was 1 mM, and 5 μCi of [^{3}H]-leucine was added to each reticulocyte reaction mixture, having a total volume of 30 μliters. Translation mixtures were incubated for 1 hr at 30°C. Reactions were stopped by plunging the mixtures on ice. For assay of acid-precipitable counts, 3-μl aliquots were removed and diluted into 0.5 ml of 3% casamino acids (Difco) in 0.1 N KOH. The mixture was incubated at 65°C for 30 min and then chilled. An equal volume of cold 25% trichloroacetic acid was added and the mixture left standing for 30 min on ice. This mixture was transferred to GF/A glass fiber discs, washed twice with 5% cold trichloroacetic acid, then with cold ethanol, dried, and counted in a scintillation mixture. The incorporation of [^{3}H]-leucine into acid-precipitable counts was linear with increasing concentration of added poly(A)$^+$ mRNA isolated from SK-N-SH neuroblastoma. Concentration of mRNA varied from 0.01 to 1.0 μg per reaction.

Polysome Preparation

Neuroblastoma cells were washed with Dulbecco's phosphate buffer and then homogenized with a Potter-Elvehjem homogenizer in 50 mM Tris/25 mM NaCl/5 mM $MgCl_2$/0.25 M sucrose buffer, pH 7.5, containing 1 mg/ml of bentonite, 0.2 mg/ml of heparin, and 1 μg/ml of cycloheximide to make a 20% (v/v) homogenate. The homogenate was centrifuged for 10 min at 25,000 x g at 4°C. The supernatant

was collected and 0.1 volume of 10% sodium deoxycholate and 10% Triton X-100 was added. Aliquots of this suspension were layered onto a discontinuous sucrose gradient (3 ml of 2.5 M sucrose and 1.5 ml of 2 M sucrose in 25 mM Tris/150 nM NaCl/5 mM $MgCl_2$, pH 7.5, containing 0.2 mg/ml of heparin and 1 g/ml of cycloheximide. Gradients were centrifuged for 17 hr at 100,000 xg in a Beckman SW-28 rotor. The 2.5 M sucrose layers containing polysomes were pooled and 1 volume of 2.5 mM Tris/150 mM NaCl/5 mM $MgCl_2$/011% Nonidet P-40 buffer, pH 7.5, containing cycloheximide and heparin was added. This solution, was dialyzed against 2 liters of the same buffer for 20-30 hr. The dialysate was then frozen in a dry ice/ethanol bath and stored at -70°C.

Monoclonal Antibody

The source of anti-acetylcholinesterase antibody was hybridoma HB-72 (ATCC), a strain produced by Fambrough et al. (3). HB-72 was maintained in our laboratory in Dulbecco's modified Eagles' medium supplemented with 10% fetal calf serum under 10% CO_2 atmosphere. Hybridoma were grown to 10^6 cells/ml in exponential phase and then transferred to serum-free medium for 25-30 hr. Approximately 1 liter of medium was then collected and centrifuged at 15,000 x g for 30 min at 4°C. The clarified medium was then brought to 50% saturation with ammonium sulfate. This suspension was centrifuged at 15,000 x g for 20 min at 4°C. The pellet was collected and suspended in 20 mM NaCl/20 mM phosphate buffer, pH 7.8, and dialyzed against the same buffer for 3 days at 4°C. The dialysate was applied to a column (1.7 x 30 cm) of DEAE-cellulose DE52 equilibrated with dialysis buffer. Protein was eluted using a 20 mM to 200 mM NaCl gradient in 20 mM phosphate buffer pH 7.8. Peak fractions were combined and concentrated by ultrafiltration using Amicon XM-50 membrane (Amicon USA, Danvers, MA 01923). The IgG fraction was adjusted to 50% (v/v) with glycerol, heparin was added to a final concentration of 0.2 mg/ml, and the antibody preparation was stored at -20°C.

IgG was tested for intactness by electrophoresis on 7.5% sodium dodecyl sulfate-polyacrylamide gels in the presence and absence of the reducing agent dithiothreitol.

Preparation of Specific mRNA

Antibody from hybridoma was used in attempts to isolate specific polysomes according to Kraus and Rosenberg (4). The polysomes prepared as described in the previous section were thawed, centrifuged to remove aggregates, and suspended in buffer to a concentration of 15 A_{260} units/ml. Polysomes were reacted for 1 hr at 4°C with purified monoclonal IgG at a ratio of 160 A_{260} units of polysomes/mg of IgG. Polysome-IgG complexes were immobilized on a protein A-Sepharose column equilibrated with 25 mM Tris/150 mM NaCl/5 mM $MgCl_2$/0.1% Nonidet P-40 buffer, pH 7.5. The column was washed thoroughly and ribosomal subunits and specific mRNA eluted from the column with a buffer containing 25 mM Tris/20 mM EDTA, pH 7.5.

The acetylcholinesterase mRNA-enriched preparation was then purified to isolate poly(A)$^+$ mRNA using an oligo-(dT)-cellulose chromatography column. The column was run without detergent or in the presence of their lithium salts, contitions used successfully in the isolation of total poly(A)$^+$ mRNA from SK-N-SH in this laboratory.

The poly(A)$^+$ mRNA enriched for acetylcholinesterase message was used as such in a reticulocyte translation system, and radiolabeled polypeptides from in vitro translation were sized by polyacrylamide gel electrophoresis and characterized by cross-reactivity with specific antibody. The results were negative.

Purification and Characterization of Monoclonal Antibody from HB-72 Hybridoma

Hybridoma HB-72 (ATCC) was used as the source of anti-human acetylcholinesterase (HACE) antibody. Growth of these cells and purification of the antibody are described in our July 1983 Annual Summary Report (5). Our purification usually started from 1 liter of serum-free medium in which the HB-72 cells were placed 25-30 hr before harvesting the medium. To obtain such a volume of medium required growing a large amount of cells.

To identify the antibody from hybridoma HB-72 as an anti-acetylcholinesterase, the following procedures were performed:

Double Immunodiffusion. A 1% agar gel in 5 mM potassium phosphate buffer, pH 7.2, containing 0.15 M NaCl was made on clean glass slides. Uniform wells were cut using a gel puncher and samples were applied to individual wells. The slides were placed in a moist chamber at 4^{o}C overnight. Then the agar plates were pressed under several layers of filter paper and a piece of heavy wooden board (10 g/cm^2) for 15 min. The gels were washed extensively in 0.15 M NaCl solution for 6-8 hr at 4^{o}C and stained with Coomassie brilliant blue. No precipitation was detected. This result was probably due to the antibody-antigen complex not being large enough to precipitate in the gel.

Immunoprecipitation. Protein A-conjugated Sepharose 4B (SPA, Pharmacia, Piscataway, NJ) was used as affinity gel. Protein A-Sepharose CL-4B is protein A covalently coupled to CL-4B by the cyanogen bromide method. 7.5 mg of SPA which contains 53 g of protein A was swollen in 0.1 M potassium phosphate buffer, pH 8.0, for 2 hr and centrifuged at 150 x g for 10 min at room temperature. The supernatant was discarded and 1 mg of crude antibody was added to the gel. The mixture was gently shaken at 4^{o}C overnight. SPA-antibody (SPA-Ab) complex was collected by centrifugation at 4^{o}C and washed three times with phosphate buffer. Fifty micrograms of HACE was added to SPA-Ab complex and gently shaken at 4^{o}C overnight. The mixture was centrifuged at 4^{o}C and the supernatant was saved for enzymatic assay. The colorimetric assay of acetylcholinesterase was used according to the method described by Ellman (6). The SPA antibody gave us 95% inhibition activity of HACE as shown by only 15% of the activity being measurable in the centrifuged supernatant after precipitation with the SPA-Ab complex.

Specificity

In order to check its specificity, an Ouchterlony immunodiffusion was performed using antibody prepared from the HB-72 hybridoma (5 mg of protein/ml), eel acetylcholinesterase (AChE) (1 mg/ml), 3% agarose, and 20 mM NaCl/20 mM PO_4 buffer, pH 7.8.

Antibody was added to the center well of a five-hole Ouchterlony. Antigen was added to four of the outer holes. The diameter of the well = 3 mm, and the distance between wells (measured from circumferences = 5 mm. Agar slides were

placed in 150-mm plastic petri dishes on top of buffer-saturated Whatman No. 1 filter paper. The dish was leveled and kept at 4°C in a cold room. After 24 hr the wells were topped off with respective Ab or antigen sample. Diffusion was allowed to run for 48 hr. Results:

HB-72 Ab X eel AChE ——> no cross-reaction

Comments

It took us much longer than expected to work up a usable preparation of this monoclonal antibody and prove its antibody specificity.

In retrospect it appears that seeking cells that produce more than normal amounts of HACE and using modern protein purification procedures might be a better approach to obtaining HACE.

TABLE I. Comparison of Acetylcholinesterase in Neuroblastoma Cell Lines

Assay Conditions: temperature, 30°C; volume, 100 μl; protein content, 0.1 mg; activity expressed in nmol/mg of protein/hr; passage number in parentheses: at least two replicates.

	Experiment 1	Experiment 2	Experiment 3
LAN-1	114 (83)	37.4 (84)	36.4 (84)
LAN-2	84 (84)	78.2 (85)	73.7 (85)
SK-N-SH	101 (30)	100.2 (30)	83.1 (31)
SK-N-MC	13 (48)	10 (50)	10.1 (50)
IMR-32	20 (64)	8.5 (65)	5.6 (65)

TABLE II. Effect of Inhibitors on Acetylcholinesterase Activity

Values give activity in percent of control. Passage number is given in parentheses.

	BW284c51 (2×10^{-5} M)	isoOMPA (10^{-4} M)
LAN-1	40 (84)	82 (83)
LAN-2	22.5 (85)	76.4 (84)
SK-N-SH	4.9 (31)	102 (30)
SK-N-MC	35 (50)	90.7 (48)
IMR-32	70.6 (65)	77.5 (64)

REFERENCES

1. Massoulie, J. and Bon, S. (1982) Molecular forms of cholinesterase and acetylcholinesterase in vertebrates. Annu. Rev. Neurosci. 5:57-106.

2. Rotondo, R.L. and Fambrough, D.M. (1979) Molecular forms of chicken embryo acetylcholinesterase in Vitro and in Vivo. J. Biol. Chem. 254:4790-4799.

3. Fambrough, D.M., Engle, A.G., and Rosenberry, T.L. (1982) Acetylcholinesterase of human erythrocytes and neuromuscular junctions: homologies revealed by monoclonal antibodies. Proc. Natl. Acad. Sci. U.S.A. 79:1078-1082.

4. Kraus, J.P. and Rosenberg, L.E. (1982) Purification of low-abundance messenger RNAs from rat liver by polysome immunoadsorption. Proc. Natl. Acad. Sci. U.S.A. 79:4015-4019.

5. Aposhian, H.V. (July, 1983) Cloning and production of human acetylcholinesterase. Contract no. DAMD 17-82-C-2142.

6. Ellman, G.L. et al. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88-95

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